



## GROWTH AND ADAPTATION OF FOUR STREPTOMYCES ISOLATES IN THE MEDIA CONTAINING PROPOXUR

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### ABSTRACT

Actinomycetes growth in the media containing carbamates is an interesting subject associated to its adaptation and metabolism behavior. In this work, four isolates of *Streptomyces* spp. had been approved to grow and demean propoxur, a carbamates pesticide commonly uses to control pest insect. The *Streptomyces* spp. cultures were incubated for seven days in the media containing propoxur, and incubated in the rotary (50 rpm) shaker-bath at 35°C. Microbial population calculated based on culture dry weight throughout separating supernatant and biomass sedimentation in the media with centrifugation work. Propoxur degradation evaluated during the isolates refined in yeast extract media containing starch (YSB) and without starch (YB), then the propoxur (0, 200, 600, 1200, and 1800 ppm) were added to the media. After a period of incubation, propoxur content in the media determined with hydrolysis to become 2-isopropoxyphenol method, and with diazotized 3-aminopyridine processes turn into azo-dye forming which became visible in 463 nm absorbance by spectrophotometric exertion. *Streptomyces* growth actions showed differently among the culture, and YSB media tendentiously stimulated microbial growth performance compared to YB media. Media restrain with starch were tending to decrease propoxur content and stimulate growth performance, and proved in some certain growth behavior among *Streptomyces* spp. Decomposing of propoxur as due to microbial growth processes were investigated through the effect on maize seedling growth performance. Growth of *Streptomyces* sp.3 isolate along cultured with propoxur in the media then used to invigorate maize seedling growth improvement. Significant consequences to seedling dry weight of maize biomass appeared after ten days growth period of seedlings.

**Keywords:** *streptomyces* spp. propoxur, degradation, plant growth improvement.

### INTRODUCTION

Degradation of residual pesticide in the tropical agriculture area turn into interest cases to converse since the pesticides are still concern to support plant yield and production in some certain places (Arbeli and Fuentes, 2007). Some of uncertainty possessions such as the limiting of efficacy and reliability of biocontrol agents, employ of chemical weed control by farmers in most regions, and use of synthetic insecticides all were unavoidable particularly in developing countries (Knutson *et al.*, 1997). In the other hand, the countries also had made different policies to determine in pesticides regulation depend on their respective interests. Altering the solution, at the moment, environmentally sound pesticides of high activity and specificity have to be used in integrated pest management systems to have availability and multiplicity of tools for maximizing flexibility, precision, and stability of pest management in agriculture (Oerke and Dehne, 2004). Accordingly, the persistence of residual pesticides makes their removal and detoxification more urgent to undertake, and in the termination bioremediation could become safety solution.

Numbers of microorganisms are able to complete interactively between chemical and physical processes for declining the target pollutants through the molecular level (Raymond *et al.*, 2001; Wiren-Lehr *et al.*, 2002; Jain *et al.*, 2005). Actinomycetes, along with other microbes' such us bacteria and fungi are known to have performing ability in biotransformation and biodegradation overcome pesticide residues in soil. Residues degradation might be increased along with organic substance alteration and caused

involving varieties of soil microbial enzymatic processes (Bricheno *et al.*, 2007; Arias-Estévez *et al.*, 2008; Rahmansyah *et al.*, 2009). Organic matters augmentation in soil served as a co-substrate during metabolism processes and will speed up the residues degradation. This metabolism process represented with inclusion in the degradation of toxic substances like the residues. Therefore, residues certainly exploited as the metabolite source by microbial degrading activities (Prescot *et al.*, 2002) and subsequently, these patterns suggested become reference and accomplishing within cultivation management to take out toxic residue in agriculture soil as the consequences of pesticide application (Soumik, 2009).

De Schrijver and De Mot (1999) investigated actinomycetes groups such as *Arthrobacter*, *Clavibacter*, *Nocardia*, *Rhodococcus*, *Nocardioides*, and *Streptomyces* perform to eliminate pesticides residues in soil. Actinomycetes had been conducted in much exploration to become practicable in decontaminated soil usage (Benimeli *et al.*, 2003; 2008). Most of *Streptomyces* genus dominated as indigenous microbes isolated from soil and had successfully capacity to growth remove and use different organochlorine pesticide (Fuentes, *et al.*, 2010). Metabolic pathways of Actinomycetes used pesticide is interesting subject to find out, particularly in *Streptomyces*. In the other hand, it was known that some soil microorganisms produce extracellular enzymes to facilitate mineralizing various complex organic compounds. Whenever, monoxygenase and dioxygenase enzymatic bacterial characteristic had been owned by mostly aerobic Actinomycetes (Larkin *et al.*, 2005).



Propoxur is a carbamates insecticide, formulated as 2-isopropoxyphenyl-N-methylcarbamate ( $C_{11}H_{15}NO_3$ ), a toxic chemical compound and use to eradicate target insect pests in agriculture practices and household purpose. Unfortunately, inappropriate use of that pesticide would accumulate residues, and under certain conditions might create adverse impact on the environment affect to non-target organism and possibly would be threatened to the human health (Cochran, 1997).

In this work, two isolates of Actinomycetes (*Streptomyces*) had been collected from soil, wherever the land had intensively used pesticides to leave pest insect disturbance in rice cultivation. Two other *Streptomyces* isolates deprived from soil in banana plantation. These isolates evaluate through their growth performance in the maintenance within the medium containing pesticides. Objective of the present study is to establish the isolates used the propoxur with their capability growth in the media with and without co-substrate of starch. Subsequently, the isolates might be shared to the agriculture management strategy for degrading pesticide residues and its occurrence in the carbamates mineralization processes due to plant nutrients availability in soil. Improving the effect of propoxur mineralization, set of test also had completed to maize plant growth performance in the potted soil which was mixed with the certain media which is the *Streptomyces* can growth in the media containing propoxur.

## MATERIALS AND METHODS

### Soil sample and isolation

Soil samples collected from two separate districts in Indonesia. One group was dig up from a rice field soil

in East Java (Semampir, Sidoarjo) which was intensively utilize chemical pesticide to eliminate undesirables' insect, and the other soil samples thrived from a marginal soil of banana plantation in Cianjur (West Java) and Way Jepara (Lampung, Southern Sumatra). In the laboratory, each one of soil subsamples were mixed thoroughly, air-dried, and non-soil material such as plant debris and stone particles take apart from the soil. The samples then crushed to pass 2 mm sieve, oven dried, and stored in sealed jars for analysis.

Isolation of *Streptomyces* achieved with one gram dried soil sample poured into 9 ml sterile aquadest which was kept in the "25 ml-volume-glass tube", and well mixed with vortex. Make a five times serial dilution (1 ml sample to 9 ml aquadest) in the glass tube containing aquadest to reach final deliberation up to  $10^{-5}$  dilution (novice-1). A hundred micro liters of novice-1 material poured onto surface of the agar media (2 g yeast extract, 10g soluble starch, 16g agar, dissolved in 1000 ml aquadest and autoclaved in 121°C at 1 atmosphere pressure for one hour) inside the petridish, trimmed with spatula, and incubated in room condition waiting for the colonies culture sprout on top of media. Subsequently, select a single and specific colony that apparently grow as *Streptomyces* colony morphology reference, then the single colony was cultured in the new media in the slant agar to be a "single isolation growth" (SIG), and keep as working collection. At last, the isolates were identified and keep as Indonesia Microbial Culture Collection (InaCC), Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences, Cibinong Science Center, West Java, Indonesia (Table-1).

**Table-1.** *Streptomyces* strains used in the study

Strain	Soil of origin	Sampling location	16 rs DNA				
			Mkr.*	sp.1	sp.2	sp.3	sp.4
<i>Streptomyces</i> sp.1	orchard (banana plantation)	Cianjur, West Java					
<i>Streptomyces</i> sp.2	orchard (banana plantation)	Lampung, Southern of Sumatra Island					
<i>Streptomyces</i> sp.3	rice field	Semampir, Sidoarjo, East Java					
<i>Streptomyces</i> sp.4	rice field	Semampir, Sidoarjo, East Java					

\*Mkr. = marker

### Growth assessment

Pick up a tiny spot of SIG on the surface of *Streptomyces* culture by using a tip of sterilized stick, sink the tip containing tiny culture into 10 ml sterilized aquadest inside the glass tube, and shake the tube with vortex to convince the culture mixed lightly; and then it was named as prepared inoculant (PI). Yeast Extract Broth (YB) media (consist of 2 g yeast extract dissolve to 1000 ml aquadest and autoclaved) and Yeast Extract Starch

Broth (YSB) media (consist of 2 g yeast extract and 10 g soluble starch, dissolve to 1000 ml aquadest and autoclaved) were preparing for growth and degradation "test media" (TM). Set a sequential of some 25 ml-bottles test (40 bottles), and filled with 10 ml TM per bottle. Add the pesticide solution (prepared with 200.000 ppm propoxur in the 20% methanol solution) in the quantity of 0, 10, 30, 60, and 90  $\mu$ l into five groups of the bottles that were already filled with TM media, respectively;



therefore, the bottles will contain 0 (control), 200, 600, 1200, and 1800 ppm propoxur, within two main groups of "with and without starch" TM media. All of bottles test inoculated with 100  $\mu$ l of PI and keep in the water shaker bath at 50 rpm, the temperature set in 35°C, and incubated for seven days incubation.

### Biomass measurement and rate of degradation

Four isolates were able to grow in the test media producing filamentous biomass. In the last seven days incubation, the biomass thrived from test media by precipitating through eppendorf tubes for centrifugation. Isolate of *Streptomyces* biomass identified to previous weight of eppendorf prior to and after collecting the samples. A hundred percent population based on the heaviest biomass weight balance to control was deliberated to be comparable calculation within the entire set of the samples in its treatment. In the other hand, differences of 2-IPP content in the media along with the treatment prior to and after incubation throughout a period of time and then calculated as the decomposing rate capability-based.

### Propoxur determination

Media containing propoxur were measured throughout alkaline hydrolysis processes turn into quantity of 2-isopropoxyphenol (2-IPP), followed by coupling the solution with 3-aminopyridine to produce colored compounds which become readable with spectrophotometer measurement in 463 nm. The method is modified from Hemasundaram and Naidu (2004). Propoxur determination was set up in the following works: (a) pick up a hundred micro liters of TM media containing propoxur from test bottles samples and dissolved into 10 ml aquadest in each tubes subsequently to each treatments; (b) add the tubes with 2.4 ml of aminopyridine solution (mixing the solutions consist of 4 part of volume of 0.1% 3-aminopyridine which is dissolve in 1% HCl; 1 part volume of 0.5% NaNO<sub>2</sub> in aqueous solution; 1 part volume of 0.5 M HCl solution; and 1 part volume of 3% sulphamic acid in aqueous solution); (c) add the tubes with 1.5 ml 2% NaOH solution to fix in alkaline (pH>10). Afterward, mixed the solution firmly and let the color become settled for 15 minutes in cool condition. Measured the color intensity, and that was the amount of 2-isopropoxyphenol quantity represent the propoxur content in the tested media.

### Degradation processes affect to maize seedling growth

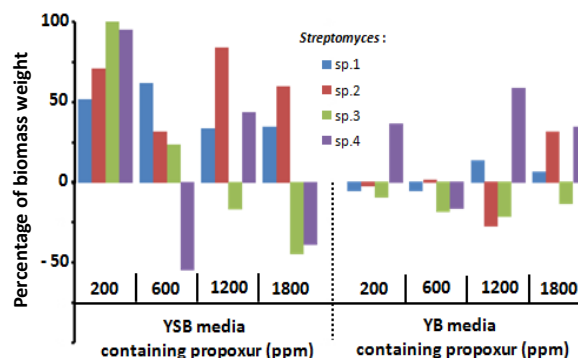
Set a sequential of some 25 ml-bottles test (20 bottles), and filled with 10 ml YSB media per bottle, and the other set filled with 10 ml of YB media. Add the pesticide solution that the bottles will become containing 600 and 1800 ppm propoxur. Set of the bottles test (containing 0, 600 and 1800 ppm propoxur) inoculated with 100  $\mu$ l of PI (*Streptomyces* sp.3) and keep in the water shaker bath at 50 rpm, in 35°C, and incubated for seven days incubation. Control preparation keep as groups of media filled with and without inoculant, as well as

negative control filled with sterilized water instead of YSB or YB.

Saw the seven days maize seedling germination for one plant per pot (apparently, a plastic cup filled with soil), and noted the initial weight of the seedlings before sawing. Fill the pots with the YSB or YB media after incubation within the propoxur degradation processes; belong to each treatment per pot. Maintain the plants to grow, and watered the pots to replace water lost normally as due to transpiration process. Seedling biomass measured after three days period of growth, and determines the final weight of plant as well. The work set into five replicates aforementioned to treatments. All the data evaluated with Stat-View for Window and Microsoft Office Excel program.

## RESULTS AND DISCUSSIONS

Normally, microorganisms were being considered and plays as the principle agents of pesticide residue elimination in soil. Four isolates of Actinomycetes in the study were able to metabolite carbamates pesticide (propoxur) as the substance improvement in the laboratory culture media. Growth response of isolates differently appears after seven days incubation, and it was depending on starch and propoxur content in the media (Figure-1). *Streptomyces* sp.1 and sp.2 had maximum reliable growth performance in the YSB media. *Streptomyces* sp.4 had the highest adaptable growth in low sole source of carbon in YB media within some certain propoxur content in the media, while 600 ppm propoxur content in media caused negative effect to its growth. All of *Streptomyces* cultures were able to grow and use carbon source available in the media further then to the simple carbon of starch.



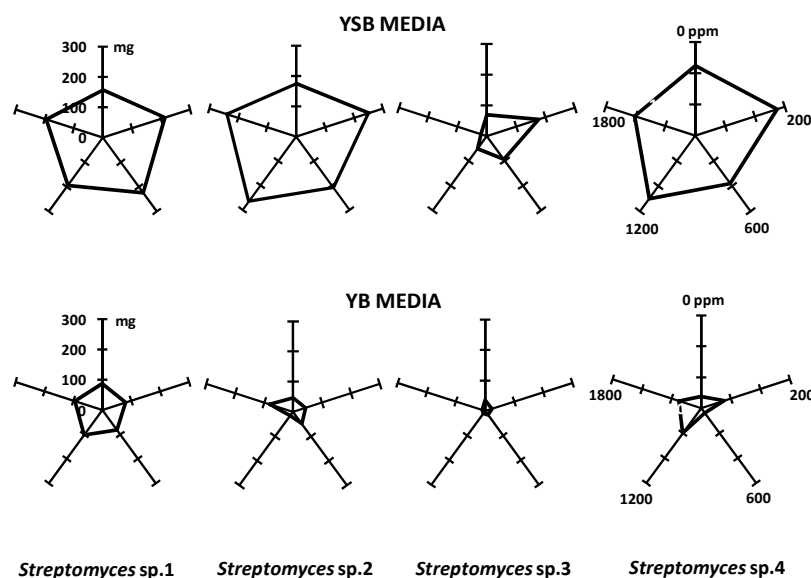
**Figure-1.** Proportion of microbial biomass growth capability become increase (upper chart) and decrease (lower chart) compared to control (0 ppm propoxur) in the media with (YSB) and without (YB) starch as carbon source augmentation in seven days incubation at 35°C.

Individual growth performance configured differently and showed their growth capability (mg biomass per 10 ml media) among the media modify with various propoxur content (Figure-2). *Streptomyces* sp.1 approximately rise double in biomass accumulation when it was grown in the media containing starch which has its



function as co-substrate in the culture. Growths performing in some other three microbial cultures were hardly appointed that all of microbes performing with fine configure due to co-substrate function of starch in the study. Propoxur will be turned into 2-IPP and methylamine through hydrolysis process with microbial hydrolyses enzymes, and then methylamine was used as carbon source in its metabolism process by the *Pseudomonas* growth (Kamanavalli and Ninnekar, 2000). Role of co-substrate acted as an inducing agent for biodegradative enzymes, and has responsibility as an electron donor for bacterial growth. The co-substrate enrichment technique using glucose was able to increase microbial decomposition capability of the benzoates and

the total amount of substitution aromatic compounds. This decomposition began after an initial lag period of 4 days and accounted for 63 to 69% degradation in 28 days (Horvath, 1973). Several media such glucose, sucrose, formate, methanol, and propionate (Movahedin *et al.*, 2006; Field and Sierra-Alvarez, 2008; Majumder and Gupta, 2008; Damianovic *et al.*, 2009) had been used to decline phenolic compound commonly used in the pesticides. Accelerate removal of pentachlorophenol by microbial fuel cells using acetate and glucose-fed for co-substrate was work properly in the laboratory experiment (Huang *et al.*, 2011). Starch in the experiment had an effective to reduce propoxur content in the media differently among the substrates for growth.



**Figure-2.** Growth configuration based on biomass weight (mg) of *Streptomyces* isolates in the media completed with starch (upper) and without starch (lower) by means of having propoxur (0, 200, 600, 1200, and 1800 ppm) in the media, for seven days incubation at 35°C.

A period of adaptation is required before the microbe able to develop and manage for establishing itself in decomposing insecticide. In the other hand, it was a natural dispossession of pesticide chemical in soil owed to microorganism metabolism activities (Arbeli and Fuentes, 2007). The phenomenon might take advantage into environmental balance, despite the fact that would be decrease to pest control efficiency. Accelerated degradation of pesticide residues has interesting implications for microbial population dynamics and turnover into soils as well as its metabolism. Microorganisms can metabolite xenobiotic compound through oxidative or reductive ways. Under favorable conditions, microorganisms can degrade that compound completely into non-toxic by-products such as carbon dioxide and water or organic acids and methane. In the reductive processes, microorganism could remove the electrophilic halogens or nitro groups (Rockne and Reddy, 2003).

Understanding the duration of the degradation is crucial approach for planning to pest management strategy. The partial data available for degradation period were varying widely among pesticides, soils with different properties, and climatic conditions. In the tropical soil, Anderson and Lafuerza (1992) found that the degradation rates of fenamiphos returned to levels into previously untreated soils after 12–16 months. Observed duration times for carbamates insecticides were usually longer then for organophosphorus insecticides and thiocarbamate herbicides (Smelt *et al.*, 1996). Low concentrations of pesticides was providing a continuous supply of substrate to the microorganisms involved in soil would make possible to other genes on the same plasmid that keep providing higher fitness to the host microorganism(s); for example, the atrazine degradation genes of *Pseudomonas* sp. strain ADP, are located on a plasmid that also contains a functional mercury resistance operon (Martinez *et al.*, 2001). In the other occurrence, the microorganism(s)



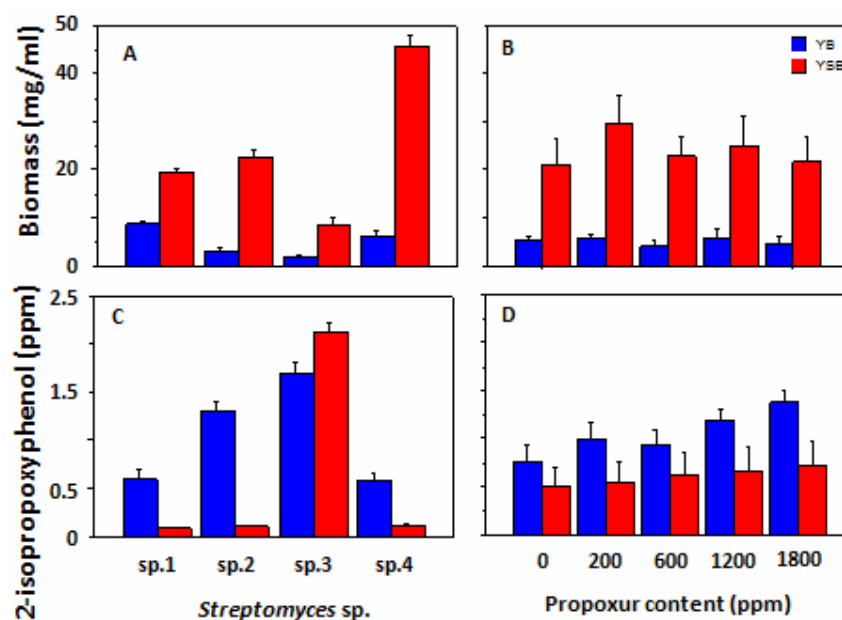
involved in the degradation are able to maintain a sufficiently high population by metabolizing other substrates.

Microbiological degradation of insecticides relies on the availability of organisms that can secrete specific degrading enzymes. Bacteria and fungi have ability to produce these enzymes under both aerobic and anaerobic conditions. Carbamates, pyrethroids and organophosphates have a common ester moiety that provides a route for bacterial-mediated enzymatic biotransformation. The enzyme groups that are able to hydrolyze these ester moieties include carboxyesterases for carbamic and carboxylic esters, and phosphotriesterases and carboxylesterases for triphosphate esters (Sogorb and Vilanova, 2002).

High concentration of 2-IPP in the media was representing low propoxur degrading capacity along with *Streptomyces* growth and its metabolism processes which were referred to increasing of all of microbial biomass (Figure-3); except for *Streptomyces* sp.3 in the study. Quantity of 2-IPP in the media without propoxur (control) were produced as its metabolites release during the microbial growth in the media. In the contrary, rate of inoculant degrading capability were commonly reduce with the escalating of propoxur content in the growth media (Figure-3B). The growth media for *Streptomyces* isolates containing 2-IPP was increase to follow propoxur content to 1800 ppm (Figure-3C), but declining degradation rate was followed by impending up of propoxur content in the growth media (Figure-3D).

Negative correlation was significant different between 2-IPP content in the growth media compared to degradation rate of isolates in YSB media ( $r=0.682 \geq p 0.01=0.408$ ;  $df=60$ ), and also significant rate when the isolate growth in YB media ( $r=0.687$ ). All isolates able to clean out propoxur in the media due to the polysaccharide (starch) enrichment in the media, and it was playing role as co-substrate occurrence in the degradation processes of YSB media; but exception was happening in *Streptomyces* sp.3 degradation performance in YB and YSB media (Figure-4).

Acclimation period was entailing the structure of viable microbial colonies that have capacity to promote and enhanced degradation. The length of the acclimation is also affected by the availability of suitable metabolic substrates for growth. Those could be a carbon source, mineral nutrient source, or both of them. The presence of alternative carbon substrates and nutrients generally increases the rate of biodegradation (Aislabie and Lloyd-Jones, 1995). This is supported by the observed increase in the rate of propoxur degradation because of *Streptomyces* spp. in the media containing starch (YSB) play as co-substrate function in this investigation. Study of the relatively primitive pathways reveals the strategies that bacteria use to evolve new capabilities and provides insight about the origins of the catabolic steps (Johnson and Spain, 2003). In the other hand, Racke *et al.* (1997) had known the phenomenon of environment functional was influencing degradation characteristic as due to altering in pH and total bacterial population improvement.



**Figure-3.** Growth and degradation characteristic of *Streptomyces* isolates in the media with starch (YSB) and without starch (YB) having propoxur and evaluate to control (0 ppm) in 7 days incubation (Error bar:  $\pm 1$  standard error of mean).

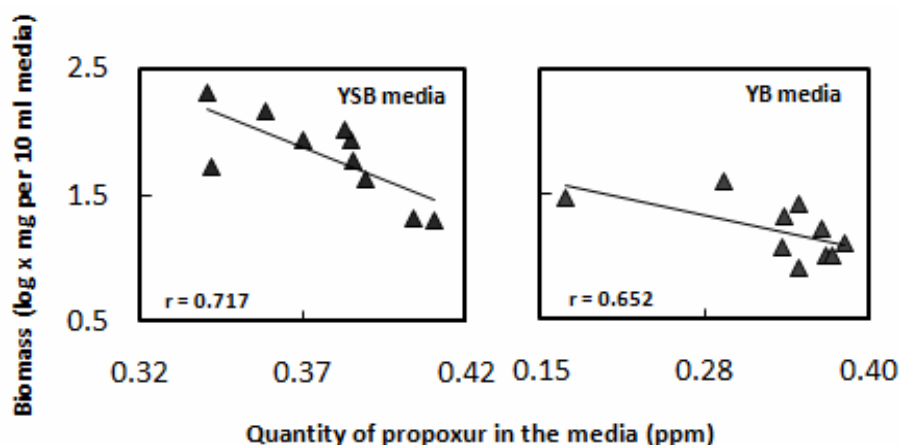
Following the phenomenon and its interpretation on the Figure-4 below, the high biomass accumulation of *Streptomyces* spp. always followed by the low quantity of

2-IPP in the media. Index of isolates biomass to 2-IPP content in YB media always less significant compared to isolates performance in YSB media, but it was exceptional



act to *Streptomyces* sp.3 which was grown in YSB media. Index value also has the same occurrence as due to propoxur substance in the growth media (see Table-1). Amount of 2-IPP found in the media without propoxur indicates that isolates have ability to hydrolysis substrate to become 2-IPP as its metabolite product. Therefore, this occasion would introduce that starch in the media positively become carbon source to lead microbial growth

in the media as co-substrate function. Analysis ratio had evaluated to isolates performance concerning within species and propoxur content in the media. Disproportionate ratio in the media without starch (YB) were tendentiously influenced by low biomass accumulation of isolates; and in the other fraction, media containing starch (YSB) were tending to the rate of propoxur degradation.



**Figure-4.** *Streptomyces* sp.3 biomass accumulation was controlled by amount of propoxur in both of the media with (left) and without (right) starch composition ( $p_{0.05} = 0.602$ ) in 7 days incubation.

**Table-1.** Index ratio of *Streptomyces* based on the degradation rate and microbial biomass at seventh day incubation

Growth media	Based on isolates				Based on propoxur content (ppm)				
	sp.1	sp.2	sp.3	sp.4	0	200	600	1200	1800
With strach (YSB)	0.09	0.14	3.04	0.05	1.35	0.49	0.65	1.90	1.07
Without strach (YB)	1.77	8.78	31.41	3.18	6.12	8.60	11.08	13.29	17.32

Measurements the propoxur containing in the media by spectrophotometric technique were practiced and compared to HPLC method, specifically in the isolates *Streptomyces* sp.3 which was sampled in the media when the propoxur degradation processes occurred. Both methods showed strong and ideals (Table-2) at each measurement value. Metabolite products disturbance occurs in the media has up to seven days incubation led to disruption of the IPP which is detected on the results of measurements in this study. Aim of this work was to validate the spectrophotometric measurement value become feasible to use in the study.

The effect of propoxur degradation by *Streptomyces* sp.3 on culture media then tested throughout maize seedling growth in the seven days after planting (7 DAP). The culture did not affect the growth rate along with 3 days seedling performance (Table-3). Seedling biomass accumulation based on changes in fresh weight recitation did not showed significant differences. The flushing effect of the microbial culture media onto seedling growth clearly different between treatments when measured in dry biomass bases at 10 DAP of seedlings.



**Table-2.** Evaluation method used in propoxur measurement for *Actinomycetes* sp.3 degrading capacity along seven days incubation in YSB media (\*\* highly significant at  $p_{0.01}=0.9587$ ; \* significant at  $p_{0.05}=0.8780$ ).

Day of observation	Correlation between spectrophotometric versus HPLC method	HPLC measurement
1	0.9895**	
2	0.9920**	
7	0.9193 *	

**Table-3.** Effect of propoxur (prox.) degradation in the media splashed into maize seedling (7 days after planting/DAP) in the potted soil (five replicates per treatment), then evaluated after three days growth period.

Treatment for maize seedling test growth	YSB media			YB media		
	Initial fresh seedling weight (mg) 7 DAP	Seedling growth rate based on fresh weight (mg/day)	Final dry seedling weight (mg) 10 DAP	Initial fresh seedling weight (mg) 7 DAP	Seedling growth rate based on fresh weight (mg/day)	Final dry seedling weight (mg) 10 DAP
<b>Inoculant and propoxur defiance into potted soil for maize seedling</b>						
Control	175.6	26.8	24.0 <sup>a</sup>	163.4	23.8	22.6 <sup>a</sup>
Media	178.8	27.8	44.0 <sup>b</sup>	177.2	27.4	33.8 <sup>abc</sup>
<b>Inoculant and propoxur compliance into potted soil for maize seedling</b>						
Inoculant ( <i>Streptomyces</i> sp.3)	174.4	27.4	43.4 <sup>b</sup>	179.8	25.8	49.0 <sup>c</sup>
Inoculant + 600 ppm. prox.	168.8	27.4	40.0 <sup>b</sup>	143.4	24.8	31.6 <sup>ab</sup>
Inoculant + 1800 ppm. prox.	155.0	21.6	20.4 <sup>a</sup>	166.8	29.2	22.6 <sup>a</sup>
Average	170.5	26.2	34.4	166.1	26.2	31.9

## CONCLUSIONS

- Four isolates of *Streptomyces* spp. were able to grow and had different mode remaining to dissimilarity in propoxur decomposing processes. The isolates become a resourceful inoculant in the detoxification processes to the pesticide residue in agriculture purposes.
- Pesticide degradation as due to microbial growth performance in this observation showed that propoxur becomes carbon source along with *Streptomyces* spp. improvement within YB media. Propoxur degradation in all treatment was being evidence for higher performance because of YSB, compared to YB for its growth media.
- In the application, carbon source as a various kind of suitable organic material supply as co-substrate was needed by *Streptomyces* spp. during growth improvement to degrade residual propoxur contaminant in soil.
- Metabolite producing processes as due to *Streptomyces* sp.3 growth activities in the media containing propoxur had affect to maize seedling dry weight differences, and so the further investigation was needed in addition to current microbial resources.



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